Modulation of iron metabolism in monocyte cell line U937 by inflammatory cytokines: changes in transferrin uptake, iron handling and ferritin mRNA

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We have investigated the effects of the pro-inflammatory cytokines interleukin 1β (IL-1β), tumour necrosis factor α (TNFα) and interferon γ (IFNγ) on the iron metabolism of the human monocytic cell line U937. Cells were treated with each cytokine for up to 24 h, and then iron uptake from diferric transferrin was determined. The intracellular distribution of this iron, the expression of the transferrin receptor and levels of mRNA for the two ferritin subunits were also studied. IL-1β, TNFα and IFNγ all decreased transferrin-iron uptake into cells, and all three cytokines had effects on the proportion of iron associated with ferritin. With TNFα there was a marked enhancement of the fraction incorporated into ferritin. Transferrin-receptor expression was diminished by TNFα and IL-1β, but not IFNγ, suggesting different effector mechanisms. Both TNFα and IFNγ increased the amount of cellular mRNA for ferritin H-chain, but not the L-chain; IL-1β affected mRNA for neither ferritin. These data demonstrate that cytokines, which can be present at high concentrations in inflammation, have the capacity to affect macrophage iron uptake, transferrin receptor expression, intracellular iron handling and the relative abundance of ferritin-subunit mRNA, and may therefore be important mediators in the observed perturbations of iron metabolism in inflammatory diseases.

INTRODUCTION

The acute-phase response of infection, inflammation or gross tissue injury is characterized primarily by an increase in hepatic synthesis of a number of serum proteins, many of which are now known to be under the control of pro-inflammatory cytokines such as interleukin 1 (IL-1β), interleukin 6 and tumour necrosis factor-α (TNFα). Chronic inflammation and malignancy are also frequently accompanied by systemic changes in iron metabolism and transport, resulting in a depression of serum transferrin and available iron occurring simultaneously with a raised intracellular iron level.

The role of cells of the macrophage lineage in inflammatory iron metabolism is pivotal. Cells of this phenotype are the primary scavengers of effete erythrocytes, and this pathway has been estimated to represent 80% of iron turnover (Finch et al., 1970). Much of the acquired iron must then be released to plasma transferrin and subsequent recycling to haematopoietic cells for new erythrocyte synthesis. The rate of iron release from within the cell *in vitro* is lower in thioglycollate- or peptone-stimulated inflammatory macrophages than in resident cells (Esparza and Brock, 1981; Alvarez-Hernandez et al., 1986; Saito et al., 1986).

One of the major determinants of cellular iron uptake is the expression of receptors for the primary iron transport protein transferrin, and in this macrophages are no exception, expressing high levels of receptors, especially after activation (Taetle and Honeysett, 1988). The expression of this glycoprotein is very tightly regulated in response to intracellular iron concentration, a mechanism which has been shown to be under translational control (Mullner et al., 1989). Although the expression of the receptor has also been linked to the cell-cycle stage (Galbraith and Galbraith, 1981), little examination of its modulation in inflammation has yet been attempted.

The role of the storage protein ferritin is also crucial in the metabolic pathways taken by iron. This is especially so in cases of inflammation. Ferritin is a hollow spherical 24-subunit polymer comprising any ratio of heavy (H-) and light (L-) subunits, which are products of independent genes. Iron is stored extremely efficiently as an Fe^{3+}-based ferrihydrite core within the molecule, where it is safely sequestered from the cytoplasm, minimizing its catalysis of free radicals (Bolann and Ulvik, 1990). A number of factors have been shown to influence the composition of ferritin, including its tissue localization (Arosio et al., 1978) and iron association (Adelman et al., 1975). Pathologically, and of particular interest, inflammation has also been shown to alter subunit composition (Schiaffonati et al., 1988), as well as causing a well-recognized increase in total quantity (Konijn and Hershko, 1977). Neither the mechanisms nor the implications of these changes have yet been thoroughly investigated.

Ferritin synthesis is also strictly modulated by iron, and, as with the transferrin receptor, the process has been shown to be translational and dependent on what is most likely the same regulatory mRNA-binding protein (the iron-response-element binding protein, IREBP) (Hentze et al., 1987; Leibold and Munro, 1988). Although this process is now relatively well understood, transcriptional modulation of ferritin and its interaction with iron-mediated modulation remain to be well characterized.

The macrophage lineage is involved in almost all aspects of the inflammatory response, primarily due to the vast array of enzymes, cytokines, free radicals and hormones of which cells may be either the source or the effector (see Nathan, 1987). In particular, macrophages are a major source of the two pro-inflammatory cytokines, TNFα (Beutler et al., 1985) and IL-1β (Yu et al., 1990), as well as possessing receptors for both cytokines (Imamura et al., 1987; Tosato and Jones, 1990). Cells are also potently primed by the T-cell product interferon γ

Abbreviations used: H-, heavy subunit of ferritin; IFNγ, interferon γ; IL-1β, interleukin 1β; IREBP, iron-response-element binding protein; L-, light subunit of ferritin; TNFα, tumour necrosis factor α.

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(IFNγ), greatly influencing their capacity to secrete enzymes, cytokines and other inflammatory mediators (Hamilton and Adams, 1987).

The actions of cytokines on macrophages therefore have the potential to modulate many aspects of their metabolism and transport of iron. However, the study of this process is complicated by the ability of this cell type to acquire iron from many sources and to respond to a range of cytokines. In order to unravel some of these interactions, we have established a relatively simple model of human macrophage iron metabolism, the uptake and processing of transferrin iron by the human monocytic cell line U937. We have examined the influence of three cytokines that are highly expressed in inflammation on the metabolism of iron by these cells, using transferrin as a source of iron for the cells, since the uptake of iron from this protein is extremely well characterized. We monitored cell iron uptake, transferrin receptor expression and iron handling, and the changes in the abundance of intracellular ferritin mRNAs. Data are presented suggesting that modulatory effects of IFNγ, TNF and IL-1β involves a complex action on all these parameters.

MATERIALS AND METHODS

U937 cells, a stable human monocytic cell line, were subcultured twice weekly in RPMI 1640 medium containing glutamine, penicillin, streptomycin and 10% (v/v) inactivated fetal-calf serum (Life Technologies, Paisley, Scotland, U.K.). Cells were regularly screened for mycoplasma and were found to be negative.

Human apotransferrin (Boehringer-Mannheim U.K., Lewes, Sussex, U.K.) was saturated with iron by using ferrous ammonium sulphate in the presence of $NaHCO_3$ (10 mM); [^{55}Fe]diferric-transferrin was prepared by the nitrilotriacetic acid method, as described by Bates and Schlabach (1973), using carrier-free $^{55}FaCl_3$ (> 50 mCi/mg of Fe) (Amersham International, Amersham, Bucks., U.K.). For receptor-binding experiments, diferric-transferrin was radio-iodinated by using Na^{125}I (Amersham) and an immobilized iodogen method (Lee and Griffiths, 1984).

Cell treatment and transferrin-Fe incubation

Cells were subcultured to 2×10^5/ml, and 5 ml volumes were subdivided into 6-well plates (Nunc; Life Technologies). Cytokine to 100 units/ml was added after 24 h, and cells were left to incubate for a further 24 h. ^{55}Fe-transferrin was then added to 12.5 μg/ml to all wells. As a control for uptake by pinocytotic or 'non-specific' mechanisms, wells were prepared to which 50 μg/ml unlabelled Fe-transferrin was added before the labelled protein. Cells were harvested hourly as described below. After 4 h a 32-fold excess of unlabelled diferric transferrin was added to all remaining wells in order to saturate the cell-surface transferrin receptors and so prevent further ^{55}Fe-transferrin uptake. Harvesting of samples of cells continued for a further 2 h. Duplicates of each sample were used in all the experiments, and were repeated on three independent occasions.

Harvesting of cells

Immediately after addition of the ^{55}Fe-transferrin, and hourly thereafter, the contents of the designated wells were transferred to pre-cooled tubes containing ice-cold PBS. Throughout the harvesting procedure, cells were kept at 4 °C or on ice from the end of incubation to lysis, to minimize proteolysis and uncontrolled transferrin uptake. Cells were pelleted at 360 $g_{av.}$ at 4 °C for 5 min, washed three times in ice-cold PBS and resuspended in 450 ml of PBS. A sample of the resuspended cells was removed to determine total iron uptake. The cells were then lysed by making to (final concns.) 0.5% Triton X-100, 0.1 mM phenylmethane sulphonyl fluoride and 0.2 mM *N*-tosyl-L-phenylalanylchloromethane in ice-cold PBS. The lysate was transferred into an autoclaved microfuge tube, repeatedly pipetted and then frozen rapidly at −70 °C until all samples could be simultaneously processed.

Recovery of ferritin iron

Labelled ferritin was recovered from cells by a method similar to that used by Alvarez-Hernandez et al. (1986) for recovery of ferritin from mouse macrophages. Briefly, lysed cells were centrifuged at 11400 $g_{av.}$ for 10 min at 4 °C and ferritin was immunoprecipitated from the supernatant by overnight incubation at 4 °C of 250 ml of supernatant with 20 μl of polyclonal anti-(human ferritin) antibody. The antiserum was raised in male New Zealand White rabbits by using highly purified human liver ferritin, which is L-rich, as is U937-cell ferritin. Pansorbin (fixed *Staphylococcus aureus*; Calbiochem Novabiochem, Nottingham, Notts., U.K.) (40 μl) was then mixed with the total solution and rotated for 2 h at room temperature. The complexes were separated from the unbound supernatant by centrifugation (2800 $g_{av.}$ for 30 s), washed, and ^{55}Fe was quantified by scintillation counting in Opto-Scint fluid (Pharmacia/LKB), by using the ^{3}H channel. Samples of the untreated cell supernatant were similarly counted for radioactivity to estimate total ^{55}Fe uptake.

Surface transferrin-receptor expression

Cells were treated for 24 h with cytokine as described above. Additional cells were similarly treated only with unlabelled human Fe-transferrin (50 μg/ml). Cells were washed twice in RPMI 1640, then resuspended and incubated for 40 min in 5 ml of pre-warmed 1% (w/v) BSA in RPMI 1640 (BSA/RPMI). This step was included to allow release of endogenous transferrin bound to receptors.

After pelleting and resuspension in 500 μl of chilled 1% BSA/RPMI, ice-cold ^{125}I-diferric-transferrin was added to 25 μg/ml to all wells. One set of replicates received a 50-fold excess of unlabelled diferric-transferrin before addition of the iodinated protein, to allow estimation of non-specific binding. All the cultures were then immediately placed on ice and incubated for 1 h in a 4 °C cold-room, with careful resuspension of the cells every 15 min. The pellets were thoroughly washed three times in ice-cold 1% BSA/PBS, and their radioactivity was then quantified by γ-radiation counting. The experiments were repeated on three separate occasions with consistent results.

Cytokine treatment of cells and RNA analysis

Cells were resuspended to 2×10^5/ml 24 h before incubation with cytokines for 24 h. Cells were harvested, pelleted and stored at −70 °C after snap-freezing in liquid N_2, or processed immediately. Total RNA was extracted by a modified acid/guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987), and immobilized on to Zeta-Bond charged nylon membrane (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.) using a Bio-Rad slot-blot unit. The mRNA-analysis experiments were repeated on at least four independent occasions.

The membrane was pre-hybridized in 7% (w/v) SDS/10 mM phosphate (pH 7.3)/1 mM EDTA for 1 h, followed by overnight incubation in the same medium containing ^{32}P-labelled cDNAs

specific for ferritin H-chain mRNA (clone 3.3 in pSp65; Murray et al., 1987), ferritin L-chain mRNA (pRLFL3, a pBR322 construct; Brown et al., 1983) or cytoplasmic β-actin (the pAL41b insert in Bluescript vector (Alonso et al., 1986). The specificity of the cDNAs was confirmed by Northern-blot analysis (results not shown). The cDNAs were labelled with ^{32}P-labelled nucleotides with a random oligonucleotide-labelling kit (Pharmacia, Milton Keynes, U.K.). The membrane was washed for 2 x 30 min at 65 °C in 5% (w/v) SDS/10 mM phosphate (pH 7.3)/1 mM EDTA, and then twice more under identical conditions in medium containing 1% (w/v) SDS. After overnight autoradiography, RNA was quantified by scanning laser densitometry. The experiments were repeated on four independent occasions and a typical experiment is portrayed.

RESULTS

Cytokines may be postulated to influence macrophage/monocyte iron metabolism through (i) an alteration of iron uptake into the cell from the external environment, (ii) by influencing the intracellular handling of iron, or (iii) by modulation of ferritin expression. These interdependent aspects were investigated by using the monocytic cell line U937.

Cellular uptake of radioactive iron from transferrin was observed over a 4 h period, during which the rate was linear (approx. 68 fmol of Fe/min per 10^6 cells in the experiment shown) (Figure 1). Cells additionally co-cultured with an excess of unlabelled diferric-transferrin showed a negligible uptake of ^{55}Fe, indicating both that successful saturation of the cell-surface transferrin receptors had occurred, and that uptake of radiolabelled iron by low-affinity ('non-specific') receptors or pinocytotic mechanisms was very low (Figure 1).

After 4 h incubation, an excess of unlabelled iron-transferrin was added to all remaining samples, to inhibit further uptake and permit study of the subsequent processing of the intracellular ^{55}Fe. As shown in Figure 1, this blocking was successful and there was negligible further uptake from the medium in any of the cultures.

Pre-treatment of the cells with any of the three cytokines tested considerably decreased the net amount of iron taken up by the cells, with the most marked difference being for TNFα. Over the first 2 h of exposure to ^{55}Fe-transferrin, TNFα-treated cells took up iron at approx. 50% of the rate of untreated cells in this experiment; after 4 h they contained only 40% of control ^{55}Fe per cell (Figure 1). IFNγ or IL-1β (at 100 units/ml) similarly decreased ^{55}Fe uptake, in both cases to approx. 65% of that in control cells. Preceding experiments indicated that these effects were not attributable to growth effects of any of the cytokines. However, one mechanism whereby the changes could have been mediated was an alteration in the number of available transferrin receptors on the cell surface. Continual recycling of receptors occurs independently of their occupancy, and a change in the expression of surface-bound receptors could be a potential modulatory mechanism.

To characterize the pathways of iron transport further, its intracellular distribution was examined by ferritin immunoprecipitation. It is established that there is complex control of the routing of the metal within the cell (Oria et al., 1988; Roberts and Bomford, 1988), and modulation of this would provide an alternative target for cytokine action.

It is notable that the amount of radiolabelled iron associated with ferritin in untreated cells increased within the time period examined, even after uptake of further extracellular ^{55}Fe-transferrin had been precluded (after 4 h; Figure 2a). This continued uptake implies the existence of a transit pool of

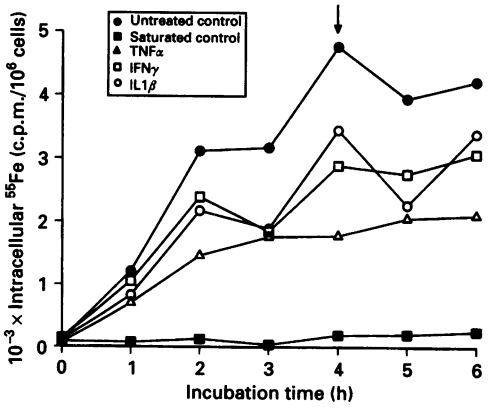

Figure 1 Total cellular uptake of ^{55}Fe

U937 cells (10^6 per well) were incubated with 100 units/ml TNFα (△), IFNγ (□) or IL1β (○), or medium alone (■, ●) for 24 h, then exposed to 12 μg/ml ^{55}Fe-transferrin. Some cultures were made 50 μg/ml with human transferrin (■) just before addition of the ^{55}Fe-transferrin. Individual cultures were harvested hourly and the radioactivity of a sample was determined. The arrow indicates the addition of a 32-fold excess of unlabelled diferric-transferrin to each remaining well.

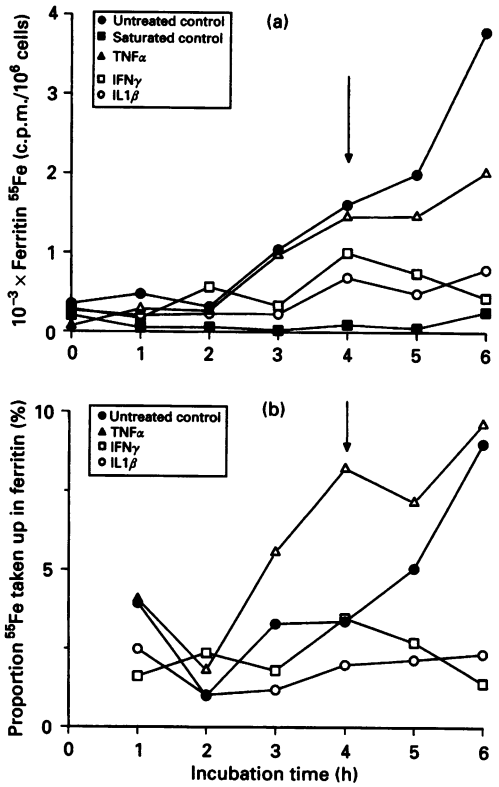

Figure 2 Iron uptake associated with ferritin

Cells were pre-treated with cytokines and allowed to take up ^{55}Fe-transferrin as described in Figure 1. Cells were then lysed in 0.5% Triton X-100 in the presence of protease inhibitors, and ferritin was immunoprecipitated with polyclonal anti-(human ferritin) antibody. After purification and washing, immune-complex-associated activity was quantified by scintillation counting. Treatment: △, TNFα; □, IFNγ; ○, IL1β; ■, 50 μg/ml human transferrin; ●, medium alone. (**a**) Total ^{55}Fe uptake; (**b**) uptake into ferritin expressed as a percentage of the total.

Table 1 Effect of cytokines on transferrin-receptor expression by U937 cells

Cells were treated with cytokine for 24 h, washed twice in RPMI 1640, then incubated for 40 min at 37 °C to clear the surface of occupied receptors. The cells were then washed in ice-cold PBS and incubated for 1 h at 4 °C in the presence of 12 μg/ml iodinated transferrin (sp. radioactivity 1100 c.p.m./ng) in 1% BSA/RPMI with regular resuspension. Specific cell-associated radioactivity was quantified after thorough washing. The receptor expression is shown as means ± S.E.M. of triplicates.

Condition	No. of transferrin receptors per cell
Control	8050 ± 454
TNFα	3418 ± 779
IFNγ	9799 ± 2309
IL1	6259 ± 1047
Human transferrin	3112 ± 154

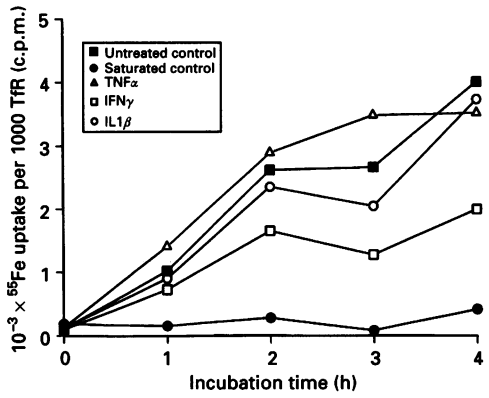

Figure 3 Effect of cytokines on iron uptake per transferrin receptor

Iron uptake by the cells from Figure 1 was combined with the receptor-expression data from Table 1 to calculate the total cellular iron uptake relative to expressed receptors.

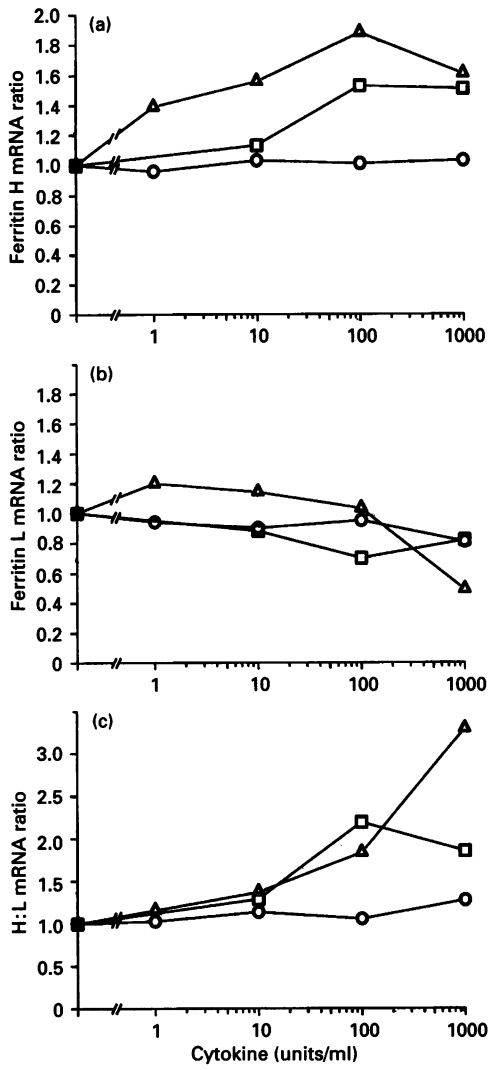

Figure 4 Cytokine-induced modulation of ferritin mRNAs

Cells were treated with cytokine for 24 h. Total RNA was extracted by acid phenol/guanidium isothiocyanate and blotted on to charged nylon. The membrane was hybridized to ^{32}P-labelled ferritin-specific cDNA probes overnight, washed and autoradiographed at −70 °C with intensifying screens. The intensity of binding was quantified by scanning laser densitometry. Changes in the amount of ferritin H-chain mRNA (**a**), ferritin L-chain mRNA (**b**) or the resultant H:L ratio (**c**) are shown, after treatment with TNFα (△), IFNγ (□ or IL-1β (○). Values are relative to untreated cells, and are corrected for RNA loading by actin hybridization in (**a**) and (**b**).

accessible material, from which iron could be further translocated into ferritin, supporting the observations by Brock and co-workers (Oria et al., 1988). It has also been shown that in the erythroleukaemic line K562, haem-pre-treated (and thus ferritin-rich) cells diverted up to 70% of iron into ferritin, as compared with only 20% in desferrioxamine-treated cells (Mattia et al., 1990).

The action of each of the three tested cytokines, however, was found to influence the intracellular handling. Whereas IL-1β and IFNγ inhibited incorporation into ferritin, the TNFα-treated cells took up into ferritin a similar amount of iron as did control cells, despite there being a greatly diminished overall uptake into the cytokine-treated cells (Figure 1). This is clearly demonstrated by Figure 2(b), in which the uptake into ferritin is expressed as a proportion of the total: the percentage of iron associated with ferritin is greatest in TNFα-exposed cells. This is a particularly interesting observation, in that it suggests that TNFα-treated cells have a more rapid capacity to respond to iron input than do untreated cells, a condition which *in vivo* could prepare cells in inflammation or immune activation for an imminent influx of iron. This effect was thus due to factors other than simply the local iron concentration; a direct effect on ferritin synthesis or stability were therefore implicated.

One obvious potential point to influence iron uptake is the expression of cell-surface transferrin receptors; the modulation of available transferrin-receptor numbers by cytokines was therefore examined (Table 1). Particularly notable was the effect of TNFα pre-treatment: transferrin-receptor expression per cell was decreased to 40% of control values. IL-1β also partially decreased transferrin-receptor expression. Normalization of total iron uptake to the number of expressed transferrin receptors clearly shows that, although a modulation of surface receptor number may be postulated as the primary or sole factor causing the TNFα- or IL-1β-mediated decreases in iron uptake, this cannot be said for IFNγ, as the corrected uptake by IFNγ-treated cells remains below that of untreated cells (Figure 3). Thus, in at

Table 2 Summary of effects of cytokines on ferritin mRNA expression

Amounts of ferritin H- and L-subunit mRNA expressed relative to untreated cells from a series of experiments in which U937 cells were treated with 100 units/ml cytokine by using the procedures described in the legend to Figure 4. The data are expressed as means ± S.E.M. ($n = 4$ for IFNγ and $n = 5$ for TNFα and IL1β).

	TNFα	IFNγ	IL1β
H	1.93 ± 0.56	1.85 ± 0.57	0.88 ± 0.12
L	1.02 ± 0.21	1.12 ± 0.28	0.91 ± 0.12
H:L	1.89 ± 0.25	1.67 ± 0.23	0.97 ± 0.05

least the case of IFNγ treatment, there is another mechanism causing the modulation of the iron uptake rate.

Control incubations in which iron was supplied from human, rather than bovine, transferrin also demonstrated a considerable decrease in bound material. As is well established, transferrin-receptor expression is inversely related to the iron content of a cell, and our work confirms previous results that the homologous protein is a considerably better iron donor than is the bovine one (Ward et al., 1982; Young and Garner, 1990).

The association of newly acquired iron with ferritin was shown to be altered by cytokines; experiments were therefore undertaken to examine the effects of cytokines on the amount of ferritin mRNAs. Both IFNγ and TNFα specifically increased the amount of ferritin H-chain mRNA relative to untreated cells, in the latter case with as little as 1 unit/ml cytokine (Figure 4a). The maximal increase due to TNFα, in this case, was 1.9 times the untreated value, and occurred at 100 units/ml cytokine (Table 2).

In contrast with TNFα, with which it shares numerous biological properties, IL-1β did not affect any systematic damage in ferritin H-chain mRNA. It is also notable that none of the tested cytokines induced a comparable change in the amount of ferritin L-chain mRNA (Figure 4b; Table 2). As a result there is an increase in the H:L ratio present in both TNFα- and IFNγ-treated cells; that in IL-1β-treated cells remains unchanged (Figure 4c; Table 2). Time-course studies indicated that the changes occurred earlier in response to TNFα (within as little as 4 h) than to IFNγ, but in both cases were still apparent up to 24 h after treatment (results not shown). As there is no evidence for selective translational control of the two species, it would be expected that such changes would be reflected in the final composition of ferritin molecules within macrophages.

DISCUSSION

The anaemia of chronic disease is a widespread and long-recognized accompaniment to many inflammatory conditions, yet little is yet known of its origin or mechanism. There is, however, good reason to suspect the involvement of macrophages in the pathogenesis, since they have a high through-put of iron and exhibit disruption of this traffic in inflammatory conditions. Inflammatory murine macrophages have a 4-fold greater rate of iron uptake than do resident cells, as well as synthesizing more ferritin protein (Birgegard and Caro, 1984). The rate of iron release from peritoneal macrophages is also slower from stimulated cells than from resident controls (Esparza and Brock, 1981; Brock et al., 1984). Such observations are in agreement with the observed iron withdrawal from the serum of patients with chronic inflammation. Similar results can be mimicked by administration of TNFα: both single and multiple inoculation of the cytokine depress serum iron *in vivo*, and TNFα treatment of macrophages *in vitro* decreases the rate of iron release with respect to control cells (Brock and Alvarez-Hernandez, 1988).

Such observations point to an important role for cytokine control of macrophage iron metabolism, and are confirmed by the work described here. The potent pro-inflammatory cytokines TNFα and IL-1β both influenced cellular iron uptake from iron uptake, as did the prime macrophage activator IFNγ. It is of particular interest that in this case the first two proteins exhibited differential activity, although they frequently share functional identity.

All three cytokines tested independently decreased the net uptake of transferrin iron in treated cells relative to untreated controls; TNFα was the most potent molecule at achieving this effect. Synergy among cytokines is observed in nearly all their modulatory activities, and it is possible that the potency of inhibiting iron uptake would be greater in combination.

Mechanisms involved in modulating transferrin iron-delivery expression in response to immune mediators have not been comprehensively investigated. There are a number of possible mechanisms whereby cytokine pre-treatment may have influenced the net iron accumulation. One of the most direct would be a modulatory effect on the number of surface-expressed transferrin receptors, a parameter investigated by using iodinated-ligand binding. These studies indicated that both IL-1β and TNFα decreased surface transferrin-receptor expression, by an amount similar to the observed decrease in iron uptake. Interestingly, however, no transferrin-receptor-expression decrease was apparent in IFNγ-treated cells, implying that the decrease in cellular iron uptake by these cells could not be attributed simply to a parallel decrease in the availability of surface transferrin receptor. Thus, although this may be postulated as the mechanism for TNFα- or IL-1β-mediated modulation of iron uptake, the data obtained indicate that it is not the means of modulation in IFNγ-treated cells.

In man, mature macrophages express considerably more transferrin-receptor mRNA and activity than do freshly isolated peripheral blood monocytes (Hirata et al., 1986; Taetle and Honeysett, 1988). Working on human macrophages obtained by maturation of human monocytes *in vitro*, Taetle and Honeysett (1988) noted an increase in surface transferrin-receptor expression on treatment with IFNγ, and yet, in agreement with work presented here, the net uptake by IFNγ-treated cells of iron from transferrin was decreased. Further experiments indicate that in IFNγ-treated cells there was a decrease in the net release of iron from transferrin into the cell.

As well as a direct change in the total number of cell-associated transferrin-receptors, caused by altered stability or synthesis of the protein or its mRNA, there are a number of other potential mechanisms. It is well known that transferrin receptors are constantly recycled whether or not they are bound to their ligand (Iacopetta et al., 1988), and as little as 20 % of cellular transferrin receptors may be expressed on the surface at any one time (Lamb et al., 1983). The rate of endocytosis is thus obviously a critical factor in the efficacy of iron uptake from the extracellular environment, and may be a target for cytokine-mediated modulation. Endocytosis is known to require both metabolic activity and an intact cytoskeleton (May et al., 1985); both these factors may be influenced by cytokine treatment.

Once iron has entered the cell, there remains considerable scope for determining its fate; primarily this will be related to its intracellular distribution. This parameter was also shown to be subject to modulation by cytokines. Although TNF treatment decreased total cellular iron uptake, it increased the proportion

of that iron entering ferritin (Figures 2a and 2b). The effect was not replicated on treatment with either IL-1β or IFNγ; indeed, very little, if any, of the freshly acquired iron was found in ferritin in the time period examined. Thus, the TNFα-stimulated incorporation of iron into ferritin is not simply a function of iron uptake, since the considerably greater rate of iron uptake by IL-1β-treated, IFNγ-treated or untreated cells meant that these cells had accumulated more iron (Figure 1).

Consistent with observations here, chelation experiments using erythroleukaemic cells indicate that ferritin-iron can be further classified into at least two compartments, being loosely associated or tightly associated iron. After removal of external Fe-transferrin, further incubation resulted in continued incorporation of iron into ferritin, implying that iron was in a transit pool of accessible material (Roberts and Bomford, 1988). The proportion of poorly accessible (non-chelatable) iron associated with ferritin similarly increased, suggesting a pathway from non-ferritin iron to loosely associated ferritin iron, to a well-sequestered non-chelatable form (Roberts and Bomford, 1988). Thus there exist varying degrees of iron association with ferritin.

It has been shown that the form in which iron is supplied to the cell is an important factor influencing its intracellular destination and properties. Iron supplied to lectin-stimulated lymphocytes as a low-molecular-mass complex (either nitrilotriacetate or citrate) cannot support their proliferation, which requires the transferrin-borne metal (Brock, 1981). Worwood et al. (1984) found differential handling, in that, whereas erythrocyte phagocytosis by human monocytes will increase both L-rich and H-rich ferritins, iron loading as Fe–nitrilotriacetic acid increased primarily the H-rich isoforms. Evidence of differential handling of iron from different sources has also been obtained in the murine macrophage line P388D_1. Of iron obtained from transferrin, 18% was partitioned into ferritin, compared with 42% of that from iron-containing soluble immune complexes (Oria et al., 1988). It would be expected that the complexes are internalized through scavenging pathways, via Fc or complement receptors. There is thus evidence of different control mechanisms operating on the two sources; the results are consistent with the hypothesis that the transferrin-uptake pathway is responsive to the iron requirements of the cell, whereas the scavenging route is primarily protective, sequestering a high proportion of its iron uptake into ferritin.

Other factors that determine intracellular compartmentalization remain unclear. However, it is known that iron-uptake rates into H-chain-rich ferritin are greater than in the L-rich protein (Wagstaff et al., 1978; Levi et al., 1988), and that H-rich ferritins isolated from human tissue contain proportionately more iron than do L-rich forms (Powell et al., 1975; Wagstaff et al., 1978). Factors which influence the H:L subunit ratio may thus be responsible for affecting the intracellular destination or sequestration of iron. It is known that synthesis of ferritin is carefully regulated at the translational level by the local iron concentration, enabling it to be very rapidly responsive to an influx of iron into the cell. However, it remained possible that cytokines were active at a previous step in the synthesis of ferritin, by affecting either transcription rates or the stability of the mRNA. The effect of cytokine treatment on ferritin H- and L-chain mRNA was therefore examined. Prior incubation of cells with either TNFα or IFNγ was found to increase the amount of ferritin H-chain mRNA, but not L-chain mRNA. These increases observed may be due to either a stabilization of the mRNA, or a rise in the rate of transcription. Work on a liver cell line has recently implicated an iron-inducible RNA-stabilizing protein in ferritin mRNA accumulation after prolonged exposure to iron (Mattia et al., 1990).

The experiments presented here indicate that the cytokine-induced changes in amount of ferritin mRNA are due to a distinct non-iron-mediated mechanism. Both TNFα and IFNγ specifically increased only the H-chain mRNA and not the L-mRNA; IL-1 did not increase either species. The changes occurred independently of intracellular iron, and indicate a selective modulation of stability or synthesis of ferritin mRNA.

To our knowledge, this is the first report of specific cytokine-mediated control of ferritin mRNAs in the monocyte/macrophage lineage, where there is considerable potential for systemic changes in iron metabolism. However, increases in ferritin mRNA have been demonstrated recently in other systems. Increased mouse ferritin H-chain mRNA has been demonstrated in muscle cells after treatment with TNFα (Torti et al., 1988; Wei et al., 1990), and shown to be independent of iron concentration (Miller et al., 1991). As in the present work, only the H- and not the L-chain mRNA increased. Interestingly, however, IFNγ had no effect in this system (Wei et al., 1990). Others have shown that ferritin H-chain mRNA is increased in thyroid cells in response to thyrotropin stimulation, and is due to an increase in transcriptional rate (Chazenbalk et al., 1990).

The precise mechanism by which mRNA is increased has not been determined in any of these systems. However, recent work has shown that cytokines frequently exert their effects by the action of intermediate enhancer proteins such as NF-κ B (Shimizu et al., 1990); the enhanced expression of at least one acute-phase protein has been shown to be directly dependent on an NF-κ B site (Edbrooke et al., 1989). Another transcriptional enhancer, AP-1, is a heterodimer of two gene products (fos and jun), both of which are induced by TNFα and directly involved in increased transcription (Brenner et al., 1989). Such a mechanism would allow for co-ordinated control by cytokines of a number of acute-phase or other proteins, possibly also including the IREBP, control systems of which are as yet unknown. Indeed, it is intriguing that the 5′ untranslated domain of the recently cloned human IREBP (Rouault et al., 1990) contains a sequence, GGACTTTAC (222–230), with high homology (Lenardo and Baltimore, 1989) to the NF-κ B recognition site (M. Fahmy and S. P. Young, unpublished work).

It has been shown that ferritins rich in H-chain subunits have an increased rate of iron uptake (Wagstaff et al., 1978; Levi et al., 1988). H-chain-rich ferritins, isolated from normal human tissues, contain proportionately more iron than the L-chain-rich forms (Powell et al., 1975; Wagstaff et al., 1978), whereas in pathological iron overload there is a reversible shift towards L-rich molecules (Powell et al., 1974). The changes in mRNA levels that we have described therefore have the potential to affect considerably the balance of iron release and retention from cells. The established control of ferritin by iron has been shown to be primarily translational, allowing the cell to respond rapidly to rising concentrations of iron. The observations of pre-translational cytokine-dependent control reported here suggest that a number of mechanisms interact to determine the final amount and form of ferritin, changes in either of which may be the cause or response to the disruption of iron metabolism in inflammatory disease.

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